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(54) Title: CELL MATRIX STIMULATED CELL ATTACHMENT AND HEMIDESMOSOME ASSEMBLY

(57) Abstract

Particular extracellular matrix proteins are disclosed, together with methods of using those proteins, and shaped articles coated with those proteins, which stimulate cell attachment and hemidesmosome formation in cells grown thereon, endocrine cell precursors grown on the matrix, including fetal pancreatic islet cell precursors, maintain biological function and differentiation.

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Cell Matrix Stimulated Cell Attachment and Hemidesmosome Assembly

Background

When organs of the body are formed, they develop in neatly organized arrays. Often, cell groups of one kind are separated from cells of another kind by flat strips of connective tissue called basement membranes. In skin, for instance, the superficial layer of epidermal cells adheres to the underlying basement membrane. This skin basement membrane acts as a barrier between the epidermal cells on the outside, and the dermal cells underneath. A similar arrangement of cells occurs in the lining of the gut.

Basement membranes have been implicated in the growth, attachment, migration, repair, and differentiation of their overlying cell populations. Three layers have been defined in basement membranes: a) the Lamina lucida, an electron microscopically clear region that resides in close approximation to the overlying cells; b) the lamina densa, an electron dense region of 20-300 nm in width; and c) the sublamina densa that contains anchoring fibrils, microfibrillar bundles and collagen fibers.

Many different types of compounds have now been localized to the basement membrane. Some of these compounds are laminin, collagen IV and heparin sulfate proteoglycans (Verrando et al. *Exp. Cell Res.* (1987), **170**:116-128). In addition, specific basement membranes include other biologically active components, such as nidogen and entactin.

The principal cell adhesion receptor that epidermal cells use to attach to the basement membrane is called $\alpha6\beta4$. This transmembrane receptor is formed by a combination of two protein moieties $\alpha6$ and $\beta4$. The $\alpha6$ and $\beta4$ proteins are derived from different genes that have been found to be part of the integrin family.

Integrins are versatile family cell adhesion receptors. So far, approximately twenty members have been discovered in the integrin family. These molecules are involved in many types of cell adhesion phenomena in the body. Integrins are

signalling molecules that can translate environmental cues into cellular instructions. Further, integrins can also transmit signals in the reverse direction, from the cell interior to the exterior. This has been illustrated in non-adherent cells, such as lymphocytes.

Stimulation of the T-cell antigen receptor, or of the CD3 complex, augments the affinity of certain integrins for their respective ligands. Unfortunately, in adherent cells, changes in the affinities of integrins have been more difficult to demonstrate. However, affinity modulation of one integrin in differentiating epidermal keratinocytes has been described by Adams et al. (*Cell*, 63: 425-435, 1990). For this reason, modifications of cell status initiated by activation or differentiation of other receptors may influence integrin affinity, and ultimately, the adhesive behavior of cells. Further, as a consequence of adhering to a surface, an integrin may actively contribute to modifying cell shape or migration.

Many epithelial cells interact with the underlying extracellular matrix via a junction called the hemidesmosome (Staehelin, (1974) *Int. Rev. Cytol.*, 39:191-278). Over the last few years there has been considerable progress in the biochemical characterization of this junction (Schwartz, et al., (1990) *Annu. Rev. Cell Biol.*, 6:461-491). The hemidesmosome, with its associated structures such as intermediate filaments and anchoring fibrils, forms an adhesion complex. Disruptions of the epithelial-connective tissue interaction are often accompanied by disruption of the hemidesmosome complex. For example, in certain blistering skin diseases such as junctional epidermolysis bullosa where epithelial cell-connective tissue interaction is abnormal, it has been proposed that there is a biochemical modification in or loss of a basement membrane zone-associated component of the hemidesmosome.

Two high molecular weight intracellular components of the hemidesmosome have been identified and characterized with the aid of antisera from patients suffering from bullous

pemphigoid. This autoimmune disease results in a disruption of the interactions between epithelial cells and connective tissue simultaneously with loss of hemidesmosome integrity (Chapman et al., (1990) *British. J. Dermatol.*, **123**:137-144). Accordingly, it was discovered that bullous pemphigoid patients were producing antibodies against hemidesmosome components. Two hemidesmosome related bullous pemphigoid (BP) antigens have been previously described (Klatte, et al., 1989).

One BP antigen is a 230 kD polypeptide that may act as an anchor for cytoskeleton elements in the hemidesmosomal plaque (Jones and Green, 1991). A second BP antigen is a type II membrane protein that possesses a collagen-like extracellular domain (Giudice, et al., 1991; Hopkinson, et al., 1992). In addition, it has been demonstrated that the interaction of the hemidesmosome with the underlying connective tissue involves the $\alpha_6\beta_4$ integrin heterodimer (Stepp, et al., (1990) *Proc. Natl. Acad. Sci. USA*, **87**:8970-8974; Jones, et al., (1991) *Cell Regul.*, **2**:427-438; Sonnenberg, et al., (1991) *J. Cell Biol.*, **113**:907-917; Kurpakus, et al., (1991) *J. Cell Biol.*, **115**:1737-1750). The $\alpha_6\beta_4$ heterodimer has been localized to hemidesmosomes along the basal surfaces of the rat bladder carcinoma cell line 804G (Jones et al. *Cell Regulation* (1991), **2**:427-438). These results suggested that integrins (e.g. $\alpha_6\beta_4$) may play an important role in the assembly and adhesive functions of hemidesmosomes.

Various prior art efforts have focused on purifying adhesion-facilitating proteins found in basement membrane. For example, Burgeson, et al., Patent Cooperation Treaty Application No. WO92/17498, disclose a protein which they call kalinin. Kalinin is said to facilitate cell adhesion to substrates; however, this material is apparently inactive with respect to hemidesmosome formation. See also, Marinkovich, et al., *J. Cell Biol.* (1992), **119**:695-703 (k-laminin); Rouselle, et al., *J. Cell. Biol.* (1991), **114**:567-576 (kalinin); and Marinkovich, et al., *J. Biol. Chem.* (1992), **267**:17900-17906 (kalinin).

Similarly, a basement glycoprotein of about 600 kD made up of polypeptides in the range of 93.5 kD to 150 kD has been identified, and is known as GB3 or nicein. See, e.g., Verrando, et al., *Biochim. Biophys. Acta* (1988); **942**:45-56 and Hsi, et al., *Placenta* (1987), **8**:209-217. None of these materials have been effective in generating formation of hemidesmosomes, either *in vitro* or *in vivo*.

When cultured on tissue culture plastic *in vitro*, most epithelial cells do not assemble bona fide hemidesmosomes despite the fact that they appear to express all of the hemidesmosomal plaque and transmembrane components mentioned above. Indeed, it is only recently that cell lines such as 804G were discovered to have the ability to readily assemble hemidesmosomes *in vitro* under regular culture conditions (Riddelle et al., (1991) *J. Cell Biol.*, **112**:159-168; Hieda et al., (1992) *J. Cell Biol.*, **116**:1497-1506). Such cells are at last allowing detailed cell and biochemical analysis of the dynamics of hemidesmosome assembly.

For instance, it has been reported that substratum-associated staining by anti-hemidesmosome antibodies is greatly diminished in 804G cell cultures that enter *in vitro* wound sites (Riddelle et al., (1992) *J. Cell Sci.*, **103**: 475-490). However, as closure of the wound became complete, anti-hemidesmosome staining along the substratum-attached surface was evident in the cells.

There are, however, many epithelial cells that do not attach to tissue culture dishes in a normal fashion, even after treatment with various growth factors. These cells do not produce normal hemidesmosomes or grow to resemble their *in vivo* phenotype. It would provide a tremendous advantage to have a system that was capable of maintaining epithelial cell growth *in vitro* wherein the cells maintained their normal phenotype.

Nearly two million Americans are afflicted with Type I (insulin-dependent) diabetes, in which the pancreas has lost its ability to secrete insulin due to an autoimmune disorder in which the insulin-secreting beta cells, found within the

islet cells of the pancreas, are destroyed. Although insulin injections can compensate for beta cell destruction, blood sugar levels can still fluctuate dramatically. The impaired ability to take up glucose from the blood results in side reactions in which toxic products accumulate, leading to complications including blindness, kidney disease, nerve damage, and, ultimately, coma and death.

Researchers have tried smaller, more frequent doses of insulin and mechanical pumps which mimic the action of the pancreas, but the results have been far from ideal. Another option, pancreatic transplant, requires major surgery and is accompanied by many complications. In addition, the limited number of donor pancreases leaves a significant number of diabetics without hope for transplantation.

The most promising option thus far is islet cell transplantation using tissue derived from either cadavers or human fetuses. This procedure has had moderate success. Among the transplants from cadavers performed worldwide, the transplanted tissue survived for a full year in about 20% of recipients. Ten of these recipients are now insulin-independent, while others have a greatly reduced need for insulin. The main problems associated with islet cell transplantation include rejection by the immune system and the autoimmune disorder which caused the disease in the first place which, if left unchecked, will also destroy the transplanted islet cells.

Islet-like cell clusters (ICCs) are composed of a heterogeneous cell population. In addition to epithelial cells which differentiate to form the endocrine, exocrine and ductal tissues, the clusters contain many stromal cells, primarily fibroblasts and endothelial cells. The presence of large numbers of stromal cells complicates the issue due to difficulties in quantitating important measures of differentiation such as insulin content per cell (Beattie et al., (1991) *J. Clin. Endocrinol. Metab.*, 73:93-98). Also, the effects of growth and differentiation factors on endocrine

precursor cells from ICCs are complicated by the presence of stromal cells.

Fetal pancreatic tissue has certain advantages over adult pancreas as a source of islet cells including its greater content of islets in proportion to its mass, its less mature endocrine cells and its greater capacity for proliferation (Voss et al., (1989) *Transplantation Proc.*, **21**:2751-2756). It is hoped that fetal islet cell transplants will dramatically reduce or eliminate diabetics' insulin dependence in a majority of patients in controlling blood sugar levels, thus minimizing the most severe diabetic complications.

Earlier attempts at culturing pancreatic islet cells were complicated by fibroblast contamination (Leach et al., (1973) *J. Endocrinol.*, 59:65-79). Although partially digested fetal pancreas has been used to produce ICCs, the clinical use of these clusters is limited because only 100-200 can be obtained per pancreas (Sandler et al., (1985) *Diabetes*, **34**:1113-1119; Otonkoski et al., (1988) *Acta. Endocrinol.*, **118**:68-76). Kover and Moore (*Diabetes*, 38:917-924, 1989) obtained 200-300 islets from a 17 week fetal pancreas, still not enough to be clinically useful. Finally, Simpson et al. (*Diabetes*, **40**: 800-808, 1991) were able to generate insulin-secreting, fibroblast-free monolayers of human fetal pancreas plated on bovine corneal matrix, although adequate numbers of cells for clinical transplantation were still not obtained. Although only a small number of cells within the clusters stain positively for the different pancreatic hormones, they differentiate efficiently into mature endocrine cells following transplantation into nude mice (Sandler et al., (1985) Diabetes, **34**:1113-1119).

Expansion of the pool of available islet cells for transplantation is highly desirable because the current technology will not produce enough cells for routine transplantation.

Summary of the Invention

One embodiment of the present invention is an article of manufacture comprising a biocompatible shaped article adapted

for use *in vivo* in a mammal, and a hemidesmosome formation-facilitating protein composition on the shaped article. Advantageously, the protein composition on the article is deposited by a tumor cell line of epithelial origin. Preferably this cell line is the rat carcinoma cell line 804G or NBTII. In another aspect of this preferred embodiment, the protein composition contains at least one of the approximately 100 kD, 135 kD, 140 kD, 150 kD, or 400 kD proteins of the extracellular matrix deposited by the cell line 804G. The article may be advantageously coated with collagen, regenerated collagen or polylactic acid and may be made of or coated with a biocompatible metal. Preferably, the metal is either stainless steel or titanium. Alternatively, the article may be made of or coated with a ceramic material. Preferably, this material is hydroxyapatite. In another aspect of this preferred embodiment, the article is made of or coated with a synthetic polymer. Preferably, this polymer is either polyester or nylon.

Another embodiment of the present invention is a composition for use in growing mammalian cells. This composition comprises the extracellular matrix protein of a mammalian cell, wherein the protein has the property of promoting hemidesmosome formation in cells contacting the protein, in a pharmaceutically acceptable carrier.

One additional embodiment of the present invention is the proteinaceous extracellular matrix proteins deposited by the cell line 804G, in substantially isolated form.

Still another embodiment of the invention is an isolated polypeptide having hemidesmosome-promoting activity consisting essentially of a 150 kD 804G matrix protein that includes the sequence of SEQ ID NO: 1.

In another preferred embodiment, there is provided a method of generating skin for allograft use comprising culturing epidermal cells onto a shaped article and growing cells under skin growth-promoting conditions.

A further embodiment of the invention is a method for increasing epidermal cell adhesion to a target surface,

comprising coating the surface with the 804G matrix. Preferably, the cells are periodontal cells. In another aspect of this preferred embodiment, the cell adhesion occurs *ex vivo*. Alternatively, the cell adhesion may occur *in vivo*.

In another aspect of the invention, there is provided a method for enhancing the growth of endocrine precursor cells by culturing the cells in the presence of one or more of the proteins found in 804G matrix. Preferably, the endocrine precursor cells are pancreatic islet cell precursors. This embodiment further provides, prior to the culturing step, enzymatically digesting fetal pancreas and incubating the digested tissue in medium until islet-like cell aggregates are formed. Preferably, the pancreas is mammalian. Most preferably, the pancreas is human and the 804G matrix proteins are solid phase proteins attached to a substrate. Another aspect of the invention provides that the 804G matrix is derived from 804G rat bladder carcinoma cells.

Another embodiment of the invention is a method for the generation of hormone-producing cells by producing expanded endocrine precursor cells and transplanting these cells into a mammal. Preferably, these cells are pancreatic islet cells, the hormone is insulin and the transplantation site is either the kidney, lung or liver.

The present invention also provides expanded endocrine precursor cells which are preferably fetal pancreatic islet precursor cells.

Detailed Description

The present invention includes the discovery that certain cell lines produce an extracellular matrix that is capable of stimulating cellular adhesion and hemidesmosome assembly in other cells subsequently grown on the matrix. One such cell line is the bladder carcinoma cell line 804G. This cell line is described by Izumi, et al., (*Cancer Res.*, 41:405-409, 1981) and is maintained in permanent collection in the laboratory of inventor Jonathan C. R. Jones, from whom the cell line is readily available. This cell line is also available from Ryoichi Oyasu, Department of Pathology, Northwestern

University Medical School, Chicago, Illinois. The 804G cell line is also maintained as a Budapest Treaty patent deposit by the American Type Culture Collection, Rockville, Maryland, USA, under accession number ATCC CRL 11555, made February 24, 1994.

A portion of one particularly useful 804G matrix protein (the 150 kD protein) has been sequenced, and that partial sequence is listed herein as SEQ ID NO:1. The present invention includes a hemidesmosome-formation-facilitating protein of 150 kD that includes the sequence of SEQ ID NO:1.

Ultrastructural data have been developed demonstrating that the 804G matrix is capable of inducing a number of cells to develop mature hemidesmosomes and attach to their growth substrate. Further, it has been discovered that the 804G matrix contains novel laminin-like molecules that participate in hemidesmosome assembly (unlike laminins and related molecules that have been purified in the prior art).

A novel matrix can now be prepared, produced by such cells as 804G cells, that can modulate the organization of hemidesmosomal antigens in unrelated cells maintained upon it. This effect appears specific to hemidesmosomal elements since adhesion plaque components do not obviously change their localization in cells maintained upon the matrix of the present invention.

To demonstrate this new discovery, evidence is provided that the murine 804G matrix was capable of inducing assembly of "mature" hemidesmosomes in human epidermal carcinoma (SCC12) cells. It can be appreciated that it is uncommon to find compounds from murine cells that have such a profound affect on human tissue. In these experiments, described in more detail below, an increased number of hemidesmosome-like structures were found in SCC12 cells maintained upon the 804G matrix as compared to control experiments wherein SCC12 cells were grown on rat tail collagen. Moreover, the majority of these hemidesmosome-like structures in the 804G matrix grown cells were in contact with the cell substrate and possessed basal dense plates. The latter structures are often used as

indicators as mature or formed hemidesmosomes (Krawczyk and Wilgram, *J. Ultrastruct. Res.*, 45:93-101, 1973).

Although methods related to production and isolation of the 804G cell matrix are specifically disclosed, it can be appreciated that any cell matrix having the ability to support cell adhesion and hemidesmosome assembly is within the scope of the present invention. Matrices from other cell types, such as the murine bladder carcinoma cell line NBTII (ATCC CRL 1655) also appear to be able to induce attachment and hemidesmosome assembly *in vitro*. The NBTII cell line is also maintained as a Budapest Treaty patent deposit by the American Type Culture Collection, Rockville, Maryland, USA, under accession number ATCC CRL 11556, made February 24, 1994. It should be noted that in the term "804G Matrix" is used to generically refer to any cell matrix with the ability to stimulate cell attachment and hemidesmosome formation.

One major use contemplated for the active components of the matrix of the present invention is in cell growth and attachment. A substrate upon which cells are to be grown is coated with the matrix or with purified hemidesmosome-promoting components thereof. The cells to be grown are then plated or applied to the substrate, and grown on the matrix. Such cells, including human cells *in vitro* and *in vivo*, will grow in an organized fashion on the substrate and will form hemidesmosomes. Hemidesmosome formation is a major advantage, because it greatly enhances the attachment of the cells to the substrate. Furthermore, it appears that the organization of cells growing on the matrix is significantly more advanced, more tissue-like, than cells grown without the matrix of the present invention.

The substrate used herein may be any desired substrate. For laboratory use, the substrate may be as simple as glass or plastic. For use *in vivo*, the substrate may be any biologically compatible material on which cells can grow. Suitable substrate materials may include shaped articles made of or coated with such materials as collagen; regenerated collagen; polylactic acid; biocompatible metals such as

stainless steel and titanium; ceramic materials including prosthetic materials such as hydroxylapatite; synthetic polymers, including polyesters and nylons; and virtually any other material to which biological molecules can readily adhere.

A specific use of the present invention is for generating skin for allograft use. Epidermal cells, for example, are seeded onto a substrate of the present invention. These cells are grown on the substrate using conventional skin growth conditions, including nutrients and growth factors. The improvement of the present invention, that is, the use of the hemidesmosome-promoting matrix on the substrate, improves such *ex vivo* growth of skin over prior art techniques that do not use that matrix.

One particular use of the present invention is to increase epidermal cell adhesion to target surfaces. For instance, prostheses for dental implantation may be treated with the 804G matrix to stimulate periodontal cell attachment. Existing teeth may similarly be coated with the matrix as a treatment for gum (junctional epithelium) disease, such as gingivitis. Where a substrate is made of a natural or synthetic bioerodible material in the form of a sheet or fabric, such as woven or bonded collagen or polylactic acid, the matrix materials may be applied to the surface thereof or mixed in with the composition. Cells (such as epidermal cells) may then be grown on the matrix *ex vivo* to form transplantable or implantable materials; alternatively, the materials may be implanted and cells may be permitted to attach *in vivo*.

In one particular embodiment of the present invention, the matrix is applied by directly growing matrix-secreting cells or by coating of matrix onto particular articles. These articles, which form particular embodiments of the present invention, include any implantable article that is placed in contact with or that extends through epithelial cells. It particularly includes, for example, articles that extend from inside an individual out through the skin. Such articles

include electrodes, other sensors, needles, cannulas, catheters, osteum equipment, sutures, bone fixing pins, screws, plates, and rods, dental implants, titanium screws and sockets for oral or other uses, plastic surgery implements including tissue expanders, and natural or artificial hair for cosmetic implantation. All of these articles can be coated with or can have incorporated into their structure one or more of the hemidesmosome-facilitating proteins of the present invention.

Also included in the scope of the present invention are ocular implants, including implantable lenses, made of any suitable material, such as silicone, natural or synthetic collagen, and the like. Furthermore, collagen contact lenses or other permanent contact lenses can be coated or impregnated with the subject proteins or matrix for permanent attachment to the cornea.

Another preferred embodiment of the present invention is the growth of increased numbers of endocrine precursor cells. Particularly interesting are pancreatic islet cell precursors. For example, fetal pancreatic islet-like cell clusters may be grown *in vitro* in the presence of one or more of the 804G matrix-type proteins for transplantation into diabetic patients. These matrix proteins will increase the yield of fetal ICCs for transplantation and will thus solve the established need for greater numbers of these cells. Since the matrix of the NBTII rat bladder carcinoma cell line is also able to promote increased epidermal cell growth, its use as a matrix for the growth of fetal pancreatic ICCs is advantageously envisioned, as is any such "804G" matrix protein, including all such proteins secreted by cell lines which are capable of promoting hemidesmosome formation in epidermal cells. In addition, the inclusion of growth factor in the ICC culture medium will further increase the yield of fetal pancreatic ICCs.

The resulting cell clusters will differentiate into functional pancreatic endocrine cells after transplantation into mammals, preferably humans, and will reduce or eliminate

the need for insulin injections. Interestingly, 804G matrix proteins have cross-species activity; even matrix derived from rat bladder carcinomas has the ability to promote growth and hemidesmosome formation in human tissue.

The 804G matrix proteins are also useful for studies concerning hemidesmosome morphogenesis, $\alpha_6\beta_4$ integrin interactions with the extracellular matrix and for functional and structural analyses of new matrix components such as the laminin B2t-like rat molecule described below. Indeed, the 804G matrix may prove to be a tool that allows definition of hemidesmosome-mediated interactions between epithelial cells and the underlying connective tissues at the molecular level.

The 804G matrix of the present invention comprises four concanavalin-binding glycosylated proteins, of approximately 135 kD, 140 kD, 150 kD, and 400 kD, and a non-glycosylated, non-concanavalin protein of about 100 kD, all of which are recognized by polyclonal antibody raised against the 804G matrix. The present invention may be practiced with the complete, active matrix from 804G cells or a functionally equivalent matrix from other cells, and may also be practiced with any one of the individual protein components of the matrix which promotes hemidesmosome formation. (Such components can be empirically determined by isolating each component and testing it in accordance with the methods described herein.)

In addition to the active matrix and the active components thereof, the present invention also includes shaped articles coated with those materials. Preferably, those shaped articles are formed of materials other than glass, and include such forms as sheets, fabrics, prostheses, metal articles, bioerodible articles, and implantable articles.

Furthermore, pharmaceutical preparations of the active matrix or its active components are contemplated. These preparations can be in any suitable form, and generally comprise the active ingredient in combination with any of the well known pharmaceutically acceptable carriers. The matrix material may be harvested (as by scraping, abrading, or

treatment with low concentrations of SDS) from surfaces on which appropriate matrix-depositing cells have been grown. Alternatively, the matrix materials may be prepared synthetically or through recombinant DNA techniques, or through purification of deposited matrix material. Those carriers can include injectable carriers, topical carriers, transdermal carriers, and the like. The preparation may advantageously be in a form for topical administration, such as an ointment, gel, cream, spray, dispersion, suspension, or paste. The preparations may further advantageously include preservatives, antibacterials, antifungals, antioxidants, osmotic agents, and similar materials in composition and quantity as is conventional. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton PA (1975).

Finally, epithelial cells of various types may be grown on the substrates or with the compositions contemplated herein.

Example 1

Preparation of 804G Cell Matrix

To begin biochemical characterization of the matrix secreted by the 804G cells, we followed the procedure of Gospodarowicz (1984). Briefly, rat bladder carcinoma 804G cells (American Type Culture Collection, Rockville, MD; ATCC CRL 11555) were maintained at 37°C in MEM with Earle's salts supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS (Gibco, Grand Island, NY). This medium contains approximately 1.9mM Ca^{2+}.

The 804G cells were grown to confluency on either plastic Petri dishes or glass coverslips. The culture medium was then discarded and the cells washed in sterile PBS. The cells were removed from their matrix by treatment for 5 minutes in sterile 20 mM NH_4OH, followed by three rapid washes with sterile distilled water.

The matrix was removed from the substrate by solubilization in 8M urea, 1% sodium dodecyl sulfate (SDS) in

10 mM Tris, pH 6.8. The 804G matrix polypeptide profile was analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using routine experimental methods known to those with skill in the art.

A preparation having approximately 20μg of the solubilized 804G cell matrix was loaded onto an acrylamide gel and electrophoresed. As a control, an extract from the removed 804G cells, having approximately 20μg per lane, was also loaded onto the acrylamide gel. Following gel electrophoreses we noted that there were three major polypeptides in the matrix preparation ranging in molecular weight from 150-135 kD. A minor polypeptide of 100 kD was also present in the matrix preparation. After PAGE, the separated polypeptides were transferred to nitrocellulose by standard well known methods. Amido black stains of the dyed protein samples were transferred to the nitrocellulose indicating a successful completion of the Western Blotting procedure.

Example 2

Concanavalin Binding to 804G Matrix

A strip of the Western Blot nitrocellulose containing separated matrix proteins was incubated with Concanavalin A. Non-specific protein binding to the matrix molecules was blocked by first incubating the strip for 30 minutes at room temperature with 2% (w/v) polyvinylpyrrolidone in PBS. Concanavalin A was added to the blocking buffer and the filter was then incubated with gentle shaking at room temperature. Horse radish peroxidase (HRP) was added to visualize Concanavalin A binding.

Four matrix polypeptides of 135, 140, 150 and 400 kD were recognized by Concanavalin A. As is known in the art Concanavalin A binding indicates that these matrix components are glycosylated. To identify proteins on the Western Blot that were specific to the matrix, we raised polyclonal and monoclonal antibodies.

Example 3

Production of Polyclonal Antibodies Against the 804G Matrix

Antiserum was prepared by injecting urea/SDS solubilized 804G cell matrix, as described above, into a rabbit by standard methods. Briefly, solubilized 804G matrix was mixed with Freund's adjuvant and injected into a rabbit. Serum was collected at three weekly intervals following one booster injection as detailed by Harlow and Lane (*Antibodies: A Laboratory Manual*, pp. 116-117 and pp. 222-223, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

The isolated polyclonal antiserum (J18) had antibodies recognizing the four 135-400 kD species that bound concanavalin A, as well as an 100 kD polypeptide. Therefore, their appears to be a non-glycosylated 100 kD species in the matrix along with four additional glycosylated polypeptides.

Following our experiments with the polyclonal antibodies, we produced monoclonal antibodies specific for the 804G matrix by the following method.

Example 4

Production of Monoclonal Antibodies Against the 804G Matrix

A mouse monoclonal IgG (5C5) against the 804G cell matrix was prepared by injecting a solubilized 804G cell matrix sample into several mice. At two and three weeks after the initial injection the mice were boosted with further 804G matrix injections. Five days following the final boost their spleens were removed and isolated spleen cells were fused with the myeloma cell line Sp2 for the production of hybridomas using standard techniques (Galfre and Milstein, (1981) *Meth. Enzymol.*, 73:3-46). Hybridoma cells producing antibody against matrix elements were selected on the basis of their immunoblotting and immunofluorescence reactivities against matrix samples. Selected hybridoma cells were cloned twice by limited cell dilution as described by Harlow and Lane (1988).

Western Blots with one of the mouse monoclonal IgG antibodies (5C5) recognized only a 150 kD and a 140 kD polypeptide in the matrix preparation. Antibody 5C5 and the J18 serum were then used in immunoprecipitation studies to investigate potential protein-protein interactions in the matrix.

Example 5

Immunoprecipitation Studies of the Matrix

Immunoprecipitation of the 804G matrix was performed using conventional methodology. In brief, the 804G matrix was treated with RIPA buffer (0.1 M Tris-HCl, pH 7.2, containing 0.15 M NaCl, 1% Triton X100, 0.1% SDS, 1% sodium deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), clarified by centrifugation, and incubated with either the rabbit serum J18 or monoclonal antibody 5C5. The resulting antibody-antigen complexes were immunoprecipitated with *Staphylococcus aureus* Protein A by methods known to those with skill in the art.

The immunoprecipitated molecules were separated by SDS-PAGE and transferred to a Western blot by the methods described in more detail above. Lanes 1 and 2 from the gel were immunoblotted with either goat anti-rabbit, or goat anti-mouse antibodies, conjugated to HRP for visualization.

The polyclonal J18 antibodies recognized similar sets of polypeptides in both the matrix and 5C5 immunoprecipitate. Major protein bands were found in both samples at 150, 140 and 135 kD. This result indicated that the J18 serum contained antibodies against all of the major proteins of the matrix.

5C5 antibodies recognized primarily 150 kD and 135 kD polypeptides in both the 804G matrix and J18 immunoprecipitate. The 5C5 antibodies apparently precipitated all of the molecular species in the matrix that were recognized by the J18 serum antibodies. In contrast, the 5C5 antibodies recognized only the 150 and 135 kD polypeptides in both the matrix preparation and the J18 serum immunoprecipitate. As the 5C5 antibodies were able to precipitate most of the matrix proteins, yet only identified two proteins on a denaturing gel, we believe that the major proteins interact and are associated with one another in their normal state. Thus, these two major 804G matrix proteins are believed to be constructed of subunits comprising the various proteins identified above.

To investigate the protein composition of the 804G matrix, we probed a Western Blot of solubilized matrix

proteins with polyclonal serum against the 400 kD and 200 kD chains of Engelbreth-Holm-Swarm (EHS) laminin.

Example 6

Western Blot of Matrix Proteins Probed with Anti-laminin Antibodies

Polyclonal antibodies against the 400 kD and 200 kD chains of EHS laminin were purchased from Collaborative Research Incorporated (Bedford, MA). A preparation of laminin (approximately 10μg per lane) and a preparation of the solubilized 804G cell matrix (approximately 20μg per lane) were denatured and run on a SDS gel, then subsequently transferred to nitrocellulose. We noted that the amido black stain used on the proteins run in lanes 1 and 2 was transferred to the nitrocellulose filter indicating that the blotting was successful.

Incubation with the HRP conjugated anti-laminin polyclonal antibodies resulted in a strong reactivity in the laminin lanes, but there was very little detectable reactivity between the laminin polyclonal antibodies and the 804G cell matrix preparation. In a related experiment, the Western Blot was immunoblotted with labeled samples of either rabbit polyclonal anti-804G serum J18 or the monoclonal antibody 5C5, respectively. These antibodies failed to recognize any laminin polypeptides, although they did recognize polypeptides in the matrix preparation as expected from previous experiments described above.

It appeared that there was little antibody cross-reactivity between laminin and the 804G matrix. For this reason, we attempted to isolate genes expressing polypeptides reactive with the J18 anti-804G antibodies.

Example 7

Isolation of Clones Corresponding to Matrix Polypeptides

A human keratinocyte lambda gt11 expression library was purchased from Clontech Labs., Inc., Palo Alto, CA and screened with the 804G matrix polyclonal serum J18 according to Huynh, et al., (*DNA Cloning: A Practical Approach, Volume I*, D. Glover, ed., IRL Press, Oxford, 1985). Antibodies

absorbed by the fusion protein products of two clones showed reactivity with the 140 kD and 100 kD molecular weight species in an 804G matrix preparation and a whole cell extract of SCC12 cells. The J18 serum was also used to screen a rat 804G expression library. Two independent clones from which antibodies to the 140 kD/100 kD polypeptide components were epitope-selected revealed over 85% identity with stretches of 94 residues in domain IV and 86 residues in domain I/II of a recently identified variant of the B2 chain of laminin that has been termed laminin B2t (Kallunki, et al., (1992) *J. Cell Biol.*, **119**:679-695). The B2t variant is not contained in EHS laminin, and therefore represents a new subunit. In addition, five clones from which antibodies to the rat 150 kD component were epitope-selected were isolated.

To further characterize positive clones, plaque lifts of nitrocellulose-bound fusion proteins were used to epitope select antibodies (Sambrook, et al., (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY). cDNA inserts were subcloned into M13 vectors and sequenced by the Sanger dideoxy chain termination method (Sanger, et al., (1977) *Proc. Natl. Acad. Sci. USA,* **74**:5463-5467). Sequence analyses were made using the GCG sequence analysis software package (University of Wisconsin Biotechnology Center, Madison, WI).

The nucleotide sequence of the 140 kDa clone revealed that it encoded a region spanning amino acids 550-810 in domain I/II of human laminin B2t. This experiment illustrates the cross-reactivity of the matrix associated polypeptides with the laminin B2t variant. The 150 kD clones encoded regions exhibiting sequence similarity to the *Drosophila* laminin A chain (Garrison et al., (1991) *J. Biol. Chem.*, **266**:22899-22904). The overall sequence identity between 294 amino acids of the rat 150 kD sequence (SEQ ID NO: 1) and amino acid residues 2365-2724 of the *Drosophila* laminin A chain (SEQ ID NO: 2) was 25%, a significant overlap considering the evolutionary difference between rat and *Drosophila*. SEQ ID NO: 1 also exhibited 21% identity to amino

acids 1634-1970 of human merosin (SEQ ID NO: 3), a laminin A isoform.

Following this experiment we attempted to ascertain the location of 804G matrix polypeptides in intact tissue samples.

Example 8

Immunofluorescence Localization of 804G Matrix Antigens in Intact Tissue

804G cells were processed for immunofluorescence using the 5C5 monoclonal and J18 polyclonal antibodies. Initially, the 804G cells were fixed and extracted for 2-3 min in 20°C acetone prior to antibody incubation. Double labeling was carried out as detailed below.

Cells on coverslips were first incubated in a mixture of primary antibodies for 1 hr at 37°C. The coverslips were extensively washed in PBS and then overlaid with the appropriate mixture of rhodamine and fluorescein conjugated secondary antibodies by well known methods. Processed tissues were viewed on a Zeiss Photomicroscope III fitted with epifluorescence optics while cultured cells were viewed on a Zeiss laser scan microscope (LSM10) equipped with Argon and HeNe lasers for dual fluorescence confocal imaging (Carl Zeiss, Thornwood, NY). As controls for the immunofluorescence analysis, cells were incubated in normal mouse, rat or rabbit IgG as well as secondary antibodies alone in order to assess staining due to non-specific antibody binding.

Both the J18 serum, 5C5 antibodies and the antibodies selected from the J18 serum using the laminin B2t fusion proteins were localized in cryo-sections of rat epithelial tissues by immunofluorescence microscopy. All of these antibody preparations show intense staining along the region of epithelial-connective tissue interaction.

All of the above experiments have been related to the structure and function of the 804G matrix. Thus far, we have determined that the 804G matrix peptides immunologically related to the B2t laminin variant, and that antibodies directed against matrix proteins have been found at the epithelial-connective tissue juncture.

One important aspect of the present invention is our discovery that the 804G matrix, described in detail above, can unexpectedly provide a substrate capable of stimulating epithelial cell growth *in vitro*. We discovered that epithelial cells grown on the 804G matrix produced hemidesmosomes, as expected from normal cells exhibiting an *in vivo* morphology. To illustrate this aspect of the invention, we performed the following experiments. Initially, we grew the SCC12 human tumor cell line on the 804G matrix to determine its potential for normal growth *in vitro*.

Example 9

Functional Analyses of Epithelial Cells Grown on the 804G matrix

Antibodies against a 230 kD plaque component of the hemidesmosome have been detailed before (Klatte et al., 1989). Monoclonal and polyclonal antibodies directed against the cytoplasmic domain (N-terminus) of a 180 kD type II membrane element of the hemidesmosome have been described in Hopkinson et al., (1992) and Riddelle et al. (1992). An antibody against the β_4 integrin subunit was purchased from Telios (San Diego, CA).

SCC12 cells were maintained on the 804G cell matrix for 24 hrs to assess the impact of the matrix on hemidesmosome protein localization in a tumor cell line that, under normal circumstances, does not assemble bona fide hemidesmosomes *in vitro*. We chose to complete our studies in 24 hrs to minimize matrix degradation and/or modification by the added cells, a possibility that Carter, et al. (1990) have discussed. Each experiment was repeated at least four times involving the analysis of more than 500 cells. As controls, the SCC12 were plated onto other matrices, such as glass and rat tail collagen. After 24 hrs the cells were processed for indirect immunofluorescence using antibodies directed against the 230 kD, 180 kD and $\alpha_6\beta_4$ integrin components of the hemidesmosome, double labelled with antibodies against the 804G cell matrix.

Cells on coverslips were first incubated in a mixture of primary antibodies for one hour at 37°C. The coverslips were

extensively washed in PBS and then overlaid with the appropriate mixture of rhodamine and fluorescein conjugated secondary antibodies. Processed tissues were viewed on a Zeiss Photomicroscope III fitted with epifluorescence optics. As controls, cells were incubated with normal mouse, rat or rabbit IgG as well as secondary antibodies alone to assess staining due to non-specific background.

In SCC12 cells maintained for 24 hrs on glass and rat tail collagen, the 230 kD, 180 kD, $\alpha_6\beta_4$ integrin subunits localized to the periphery of the cells along their substratum attached surfaces. The staining sometimes resembled a fuzzy band surrounding the cell periphery, or linear streaks near the cell edges (see also Hopkinson, et al., 1991). Anti-matrix antibodies in the J18 serum generated a diffuse staining along the region of cell-substrate interaction in cells maintained on rat tail collagen, with no obvious correlation to the staining generated by the hemidesmosomal antibody probes. The reactivity of J18 antibodies with the SCC12 cells by immunofluorescence is consistent with the positive immunoblotting reactivity using antibodies selected from the J18 serum by the human laminin B2t fusion proteins. Since antibodies in the J18 serum failed to recognize rat tail collagen alone, our results provide some indication concerning the matrix that the SCC12 cells themselves secrete.

In SCC12 cells maintained on the 804G cell matrix, the 230 kD, 180 kD and $\alpha_6\beta_4$ integrins show a dramatically different pattern of distribution compared with that observed in cells maintained on rat tail collagen or glass. The patterns that these hemidesmosomal antibodies generate are similar to that seen in 804G cells processed for immunofluorescence using the same antibodies, as described above. Furthermore, this staining, in most instances, appears coincident with those patterns generated by antibodies in the whole J18 serum.

In addition, 5C5 antibodies or those J18 antibodies epitope selected from the laminin B2t fusion proteins were also localized in SCC12 cells maintained on the 804G matrix. The distribution of these antibodies compared with that of the

230 kD hemidesmosomal plaque component. It should be noted that the 230 kD antigen distribution in the SCC12 cells mirrors that of the staining generated by the 5C5 and epitope selected antibodies.

Immunoblotting analyses were undertaken to examine whether there was a change in the amounts of both the 230 kD and 180 kD hemidesmosomal components in SCC12 cells maintained on 804G cell matrix for 24 hrs compared to SCC12 cells maintained for the same length of time on other matrices. There was no apparent difference in the quantity of both the 230 kD and 180 kD polypeptides in SCC12 cells maintained on the various matrices as assessed by this procedure.

In contrast to hemidesmosomal components, the $\alpha_5\beta_1$ integrin complex, a component of the microfilament associated-adhesion plaque (Burridge, et al. 1988), localize primarily at the peripheral cell-substratum associated surface of SCC12 cells regardless of whether it is maintained on rat tail collagen or the 804G cell matrix.

Our studies of epithelial cell growth on the 804G matrix were not confined to SCC12 cells. Normal human keratinocytes (derived from human foreskins), HaCaT (immortalized cells), and SCC13 cells also exhibited almost identical responses when grown on the 804G matrix in comparison to the SCC12 cells discussed above. In each of these cell types, growth on the 804G matrix led to a redistribution of integrins and mature hemidesmosome formation.

In addition, experiments similar to those described above have been performed on the matrix produced by the NBTII cell line (American Type Culture Collection, Rockville, MD; ATCC CRL 11556). The results from these experiments are virtually identical to those illustrated for the 804G matrix. Cells grown on the NBTII matrix were stimulated to form mature hemidesmosomes and redistribute intracellular integrins.

To further investigate the effect of growing epithelial cells on the 804G matrix, we examined SCC12 cells under the electron microscope.

Example 10

Electron Microscopic Examination of the Impact of the 804G Cell Matrix on Hemidesmosome Assembly in SCC12 Cells

SCC12 cells were fixed and processed for electron microscopy as described elsewhere (Riddelle, et al., 1991). Thin sections of cells were made perpendicular to their substrate, placed on 300 mesh electron microscope grids (Tousimis Corp., Rockville, MD), stained and then viewed at 60 kV in a JEOL 100CX electron microscope.

SCC12 cells maintained for 24 hrs on either rat tail collagen or the 804G matrix were examined by conventional electron microscopy. This procedure involved analyzing thin sections of the SCC12 cells cut perpendicularly to their substrate at intervals of 10 microns through a population of cells. By assessing sections at this distance apart we avoided the possibility of observing the same hemidesmosome more than once.

In SCC12 cells maintained for 24 hrs on rat tail collagen, hemidesmosome-like structures were observed towards the cell periphery. In 17 SCC12 cells incubated on rat tail collagen we observed 9 hemidesmosome-like structures, none of which possessed a basal dense plate. This count was made over a distance of 306 microns (i.e. 1 hemidesmosome-like structure/34 microns of the ventral surfaces of SCC12 cells). The close apposition of three hemidesmosome-like structures was seen in one micrograph, however, this was highly unusual. In many basal profiles of SCC12 cells on rat tail collagen no hemidesmosomes were observed.

In contrast, 103 hemidesmosome-like structures, of which 92 possessed basal dense plates, were observed in cross sectional profiles of SCC12 cells incubated on the 804G matrix. These observations were made over a distance of 504 microns (i.e., 1 hemidesmosome-like structure/4.9 microns of SCC12 ventral surface). Unlike the "rudimentary" hemidesmosomes seen on cells incubated with rat tail collagen, these hemidesmosome-like structures were not confined to the periphery of the cell, but also were found underlying the

nucleus. These SCC12 cells also appeared to possess tufts of intermediate filaments associated with their cytoplasmic face.

In addition to electron microscopy of SCC12 cells, we looked for hemidesmosome assembly in Human Keratinocytes, HaCaT cells, and SCC13 cells. As reported above in relation to immunofluorescence experiments, each of these other mammalian epithelial cells began redistributing integrins and forming mature hemidesmosomes. Our electron microscope studies revealed significant similarities in the affect of the 804G matrix on SCC12 cells, Human Keratinocytes, HaCaT cells, and SCC13 cells.

To demonstrate that the 804G cell matrix could retain its ability to induce changes in epithelial cells after solubilization, we coated glass coverslips with solubilized matrix elements.

<u>Example 11</u>

<u>Photolithography with 804G Matrix Elements</u>

To determine whether an isolated matrix sample could retain its ability to induce changes in hemidesmosomal and integrin localization 804G cells were grown and removed from their matrix as described above. A mild SDS buffer (RIPA) was used to solubilize and remove the matrix from its growth substrate. Following solubilization in RIPA buffer, the matrix elements were dialyzed extensively against phosphate buffered saline and then coated in a microscopic pattern onto glass coverslips using a photolithographic technique described by Hockberger et al. (*J. Neurosci.* (1988) **8(11)**: 4098-4120).

Briefly, a clean coverslip was first spin-coated with "photoresist". A mask was placed on top of the photoresist layer followed by illumination with UV light. At all of the points not covered by the mask the photoresist was UV cross-linked to the glass coverslip. Dialyzed 804G matrix elements were then added to the coverslip and bound along the entire surface of the coverslip. The photoresist and its bound matrix elements were removed from the non-UV linked areas of the coverslip by acetone treatment. A defined pattern of 804G

matrix elements, configured as the inverse of the mask, was retained for further examination.

In immunofluorescence studies using our matrix polyclonal antiserum, we demonstrated that SCC12 cells grown on these coverslips form hemidesmosomes in formations corresponding to the deposited pattern of 804G elements. Remarkably, the location of β_4 integrins on SCC12 cells grown on these coverslips also followed the deposited matrix patterns. This indicated that the matrix maintained its functionality following mild SDS denaturation and deposit onto a solid substrate. By following this protocol, other solid substrates could be coated with the 804G matrix to stimulate hemidesmosomal formation in epithelial cells.

Thus, we have demonstrated that the 804G cell matrix is able to induce attachment and hemidesmosome assembly in many types of mammalian cells.

Example 12

Expansion of fetal pancreas islet cells *in vitro*

Human fetal pancreases are minced into 1 mm pieces in cold Hanks' balanced salt solution (HBSS) and digested with collagenase P by shaking vigorously for 15 min in a water bath at 37°C. After several washes at 4°C with HBSS, the digested tissue is washed with cold HBSS and placed into petri dishes in RPMI-1640 medium containing 10% pooled human serum and antibiotics for three days. Optionally, a growth factor is present during this procedure.

Approximately 50 ICCs of uniform size (50-75 μM diameter) and homogeneous translucent appearance are hand picked and plated on tissue culture dishes coated with either 804G matrix or bovine corneal matrix in RPMI-1640 containing 15% horse serum, 5% FCS, antibiotics and, optionally, a growth factor. ICCs attach overnight and monolayer formation is generally initiated by 24 hours. A significant increase is observed in the number of ICCs plated on 804G matrix compared to either no matrix or to bovine corneal matrix.

To determine whether these fetal endocrine cells are capable of differentiating into insulin-producing cells *in vivo*, ICCs are transplanted as described below.

Example 13

Transplantation of ICCs into nude mice

ICCs from Example 12, cultured on 804G matrix, are transplanted under the kidney capsule of athymic nude mice (approximately 500 ICCs per mouse) and the grafts are analyzed after 3 months. An increased level of human C-peptide, released into the blood after processing of the insulin precursor molecule, is detected in the blood of grafted animals by radioimmunoassay after an intraperitoneal glucose challenge indicating that the grafted cells are able to produce insulin. In addition, immunocytochemistry of graft cells using an antibody to insulin indicates that the precursor cells differentiate into insulin-producing cells.

Example 14

Transplantation of ICCs into diabetic patients

Human diabetes patients are administered a number of fetal ICCs to be optimized in clinical studies. Presumably, this number will be close to that used for adult-derived cells, approximately 2-8 x 10^5, either by implantation under the kidney capsule or by direct injection into the liver. In addition, transplantation in other ectopic organ locations is also contemplated. C-peptide production and blood glucose levels are monitored over several months to determine whether transplanted endocrine precursor cells have differentiated into insulin-producing cells. The patients are still administered insulin during the monitoring period.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: DESMOS, Inc., 7720-B El Camino Real, Carlsbad, California, USA

(ii) TITLE OF INVENTION: Cell Matrix Stimulated Attachment and Hemidesmosome Assembly

(iii) NUMBER OF SEQUENCES: 3

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(B) FILING DATE: 5 April 1993

(A) APPLICATION NUMBER: 08/152,460
(B) FILING DATE: 12 November 1993

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 295 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: 150 kD

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Gln Thr Leu Tyr Asn Asn Ile Asp Thr Thr Ile Gln Asn Ala Lys Glu
            20                  25                  30

Leu Asp Met Lys Ile Lys Asn Ile Leu Thr Asn Val His Ile Leu Leu
        35                  40                  45

Lys Gln Ile Ala Arg Pro Gly Gly Glu Gly Met Asp Leu Pro Val Gly
    50                  55                  60

Asp Trp Ser Arg Glu Ser Ala Glu Arg His Gly His Val Ala Glu Ser
65                  70                  75                  80

Arg Gly Arg Asp Phe Lys Lys His Leu Gln Glu Ala Glu Ala Gln Lys
                85                  90                  95

Met Glu Ala Gln Leu Leu Leu Asn Arg Ile Arg Thr Trp Leu Glu Ser
            100                 105                 110

His Gln Val Glu Asn Asn Gly Leu Leu Lys Asn Ile Arg Asp Ser Leu
        115                 120                 125

Asn Asp Tyr Glu Ala Lys Leu Gln Asp Leu Arg Ser Val Leu Gln Glu
    130                 135                 140

Ala Ala Ala Gln Gly Lys Gln Ala Thr Gly Leu Asn His Glu Asn Glu
145                 150                 155                 160

Gly Val Leu Gly Ala Ile Gln Arg Gln Met Lys Glu Met Asp Ser Leu
                165                 170                 175

Lys Lys Tyr Leu Thr Glu His Leu Ala Thr Ala Asp Ala Ser Leu Leu
            180                 185                 190

Gln Thr Asn Ser Leu Leu Gln Arg Met Asp Thr Ser Gln Lys Glu Tyr
        195                 200                 205

Glu Ala Trp Gln Ile Asp Ile Ser Leu Glu Gln His Pro Val His Asn
    210                 215                 220

Cys Leu Leu Arg Leu Thr Leu Arg Gln Asp Leu Ile Asp Leu Asn Phe
225                 230                 235                 240

Ser Phe Ser Val Pro Gln Val Val Asp Thr Arg Gln Leu Ala Ile Tyr
                245                 250                 255

Asn Arg His Ala Tyr Val Val Leu Gly Gly Ile Leu Val Ser Lys Val
            260                 265                 270

His Tyr Lys His Cys Pro Thr Cys Leu His Ser Leu Leu Ser Leu Val
        275                 280                 285

Phe Gly Gly Thr Lys Thr Tyr
    290                 295
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: laminin A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Lys Phe Asp Thr Val Ser Glu Gln Lys Leu Gln Ala Glu Lys Asn Ile
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Lys Asp Ala Gly Asn Phe Leu Ile Asn Gly Asp Leu Thr Leu Asn Gln
            20                  25                  30

Ile Asn Gln Lys Leu Asp Asn Leu Arg Asp Ala Leu Asn Glu Leu Asn
        35                  40                  45

Ser Phe Asn Lys Asn Val Asp Glu Glu Leu Pro Val Arg Glu Asp Gln
    50                  55                  60

His Lys Glu Ala Asp Ala Leu Thr Asp Gln Ala Glu Gln Lys Ala Ala
65                  70                  75                  80

Glu Leu Ala Ile Lys Ala Gln Asp Leu Ala Ala Gln Tyr Thr Asp Met
                85                  90                  95

Thr Ala Ser Ala Glu Pro Ala Ile Lys Ala Ala Thr Ala Tyr Ser Gly
            100                 105                 110

Ile Val Glu Ala Val Glu Ala Ala Gln Lys Leu Ser Gln Asp Ala Ile
        115                 120                 125

Ser Ala Ala Gly Asn Ala Thr Asp Lys Thr Asp Gly Ile Glu Glu Arg
    130                 135                 140

Ala His Leu Ala Asp Thr Gly Ser Thr Asp Leu Leu Gln Arg Ala Arg
145                 150                 155                 160

Gln Ser Leu Gln Lys Val Gln Asp Asp Leu Glu Pro Arg Leu Asn Ala
                165                 170                 175

Ser Ala Gly Lys Val Gln Lys Ile Ser Ala Val Asn Asn Ala Thr Glu
            180                 185                 190

His Gln Leu Lys Asp Ile Asn Lys Leu Ile Asp Gln Leu Pro Ala Glu
        195                 200                 205
```

Ser Gln Arg Asp Met Trp Lys Asn Ser Asn Ala Asn Ala Ser Asp Ala
210 215 220

Leu Glu Ile Leu Lys Asn Val Leu Glu Ile Leu Glu Pro Val Ser Val
225 230 235 240

Gln Thr Pro Lys Glu Leu Glu Lys Ala His Gly Ile Asn Arg Asp Leu
245 250 255

Asp Leu Thr Asn Lys Asp Val Ser Gln Ala Asn Lys Gln Leu Asp Asp
260 265 270

Val Glu Gly Ser Val Ser Lys Leu Asn Glu Leu Ala Glu Asp Ile Glu
275 280 285

Glu Gln Gln His Arg Val Gly Ser Gln Ser Arg Gln Leu Gly Gln Glu
290 295 300

Ile Glu Asn Leu Lys Ala Gln Val Glu Ala Ala Arg Gln Leu Ala Asn
305 310 315 320

Ser Ile Lys Val Gly Val Asn Phe Lys Pro Ser Thr Ile Leu Glu Leu
325 330 335

Lys Thr Pro Glu Lys Thr Lys Leu Leu Ala Thr Arg Thr Asn Leu Ser
340 345 350

Thr Tyr Phe Arg Thr Thr Glu Pro
355 360

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 337 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: merosin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Leu Lys His Leu Leu Ser Pro Gln Arg Ala Pro Glu Arg Leu Ile
1 5 10 15

Gln Leu Ala Glu Gly Asn Leu Asn Thr Leu Val Thr Glu Met Asn Glu
20 25 30

Leu Leu Thr Arg Ala Thr Lys Val Thr Ala Asp Gly Glu Gln Thr Gly
35 40 45

```
Gln Asp Ala Glu Arg Thr Asn Thr Arg Ala Lys Ser Leu Gly Glu Phe
    50                  55                  60

Ile Lys Glu Leu Ala Arg Asp Ala Glu Ala Val Asn Glu Lys Ala Ile
65                  70                  75                  80

Lys Leu Asn Glu Thr Leu Gly Thr Arg Asp Glu Ala Phe Glu Arg Asn
                85                  90                  95

Leu Glu Gly Leu Gln Lys Glu Ile Asp Gln Met Ile Lys Glu Leu Arg
            100                 105                 110

Arg Lys Asn Leu Glu Thr Gln Lys Glu Ile Ala Glu Asp Glu Leu Val
        115                 120                 125

Ala Ala Glu Ala Leu Leu Lys Lys Val Lys Lys Leu Phe Gly Glu Ser
    130                 135                 140

Arg Gly Glu Asn Glu Glu Met Glu Lys Asp Leu Arg Glu Lys Leu Ala
145                 150                 155                 160

Asp Tyr Lys Asn Lys Val Asp Asp Ala Trp Asp Leu Leu Arg Glu Ala
                165                 170                 175

Thr Asp Lys Ile Arg Glu Ala Asn Arg Leu Phe Ala Val Asn Gln Lys
            180                 185                 190

Asn Met Thr Ala Leu Glu Lys Lys Lys Glu Ala Val Glu Ser Gly Lys
        195                 200                 205

Arg Gln Ile Glu Asn Thr Leu Lys Glu Gly Asn Asp Ile Leu Asp Glu
    210                 215                 220

Ala Asn Arg Leu Ala Asp Glu Ile Asn Ser Ile Ile Asp Tyr Val Glu
225                 230                 235                 240

Asp Ile Gln Thr Lys Leu Pro Pro Met Ser Glu Glu Leu Asn Asp Lys
                245                 250                 255

Ile Asp Asp Leu Ser Gln Glu Ile Lys Asp Arg Lys Leu Ala Glu Lys
            260                 265                 270

Val Ser Gln Ala Glu Ser His Ala Ala Gln Leu Asn Asp Ser Ser Ala
        275                 280                 285

Val Leu Asp Gly Ile Leu Asp Glu Ala Lys Asn Ile Ser Phe Asn Ala
    290                 295                 300

Thr Ala Ala Phe Lys Ala Tyr Ser Asn Ile Lys Asp Tyr Ile Asp Glu
305                 310                 315                 320

Ala Glu Lys Val Ala Lys Glu Ala Lys Asp Leu Ala His Glu Ala Thr
                325                 330                 335

Lys
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WE CLAIM:

1. An article of manufacture, comprising:

a biocompatible shaped article adapted for use *in vivo* in a mammal; and

a hemidesmosome formation-facilitating protein composition on said shaped article.

2. The article of Claim 1, wherein said protein composition is deposited by a tumor cell line of epithelial origin.

3. The article of Claim 2, wherein said tumor cell line is the rat carcinoma cell line 804G.

4. The article of Claim 2, wherein said tumor cell line is the rat bladder cancer cell line NBTII.

5. The article of Claim 1, wherein said protein composition contains at least one of the approximately 100 kD, 135 kD, 140 kD, 150 kD, or 400 kD proteins of the extracellular matrix deposited by the cell line 804G.

6. The article of Claim 5 in the form of a sheet, further comprising epidermal cells cultured on said matrix.

7. The article of Claim 1, further comprising coating said article with either collagen, regenerated collagen or polylactic acid.

8. The article of Claim 1, wherein said article is made of or coated with a biocompatible metal.

9. The article of Claim 8, wherein said metal is either stainless steel or titanium.

10. The article of Claim 1, wherein said article is made of or coated with a ceramic material.

11. The article of Claim 10, wherein said ceramic material is hydroxyapatite.

12. The article of Claim 1, wherein said article is made of or coated with a synthetic polymer.

13. The article of Claim 12, wherein said polymer is either polyester or nylon.

14. A composition for use in growing mammalian cells, comprising:

extracellular matrix protein of a mammalian cell, which protein has the property of promoting hemidesmosome formation in cells contacting said protein; in a pharmaceutically acceptable carrier.

15. One or more of the proteinaceous extracellular matrix proteins deposited by the cell line 804G, in substantially isolated form.

16. An isolated polypeptide consisting essentially of a 150 kD protein that includes SEQ ID NO: 1 and has the ability to facilitate hemidesmosome formation in epithelial cells cultured thereon.

17. A method of generating skin for allograft use comprising:

culturing epidermal cells onto the article of Claim 1; and

growing said cells under skin growth-promoting conditions.

18. A method for increasing epidermal cell adhesion to a target surface, comprising coating said surface with at least one of the approximately 100 kD, 135 kD, 140 kD, 150 kD, or 400 kD proteins of the extracellular matrix deposited by the cell line 804G.

19. The method of Claim 18, wherein said cells are periodontal cells.

20. The method of Claim 18, wherein said cell adhesion occurs *ex vivo*.

21. The method of Claim 18, wherein said cell adhesion occurs *in vivo*.

22. A method for growing endocrine precursor cells, comprising the step of culturing endocrine precursor cells in the presence of 804G matrix protein capable of promoting enhanced growth of said cells.

23. The method of Claim 22, wherein said endocrine precursor cells are pancreatic islet cell precursors.

24. The method of Claim 23, further comprising the steps prior to the culturing step of:

enzymatically digesting fetal pancreas;

incubating the digested pancreas in cell culture medium until islet-like cell aggregates are formed.

25. The method of Claim 24, wherein said pancreas is from a mammal.

26. The method of Claim 25, wherein said pancreas is human.

27. The method of Claim 22, wherein said 804G matrix proteins are solid phase proteins attached to a substrate.

28. The method of Claim 22, wherein said matrix is derived from 804G rat bladder carcinoma cells.

29. A method for the generation of hormone-producing cells, comprising the steps of:

providing expanded endocrine precursor cells according to the method of Claim 1; and

transplanting said expanded endocrine precursor cells into a mammal.

30. The method of Claim 28 wherein said cells are pancreatic islet cells.

31. The method of Claim 28 wherein said hormone is insulin.

32. The method of Claim 31 wherein said cells are transplanted into or adjacent to the kidney, lung or liver.

33. Endocrine precursor cells prepared in accordance with Claim 22.

34. The cells of Claim 33, which are fetal pancreatic islet precursor cells.

AMENDED CLAIMS

[received by the International Bureau on 24 August 1994 (24.08.94); original claims 23,34 cancelled; original claims 22,27,33 amended; remaining claims unchanged; and renumbered from 1-32 (2 pages)]

extracellular matrix protein of a mammalian cell, which protein has the property of promoting hemidesmosome formation in cells contacting said protein; in a pharmaceutically acceptable carrier.

15. One or more of the proteinaceous extracellular matrix proteins deposited by the cell line 804G, in substantially isolated form.

16. An isolated polypeptide consisting essentially of a 150 kD protein that includes SEQ ID NO: 1 and has the ability to facilitate hemidesmosome formation in epithelial cells cultured thereon.

17. A method of generating skin for allograft use comprising:

culturing epidermal cells onto the article of Claim 1; and

growing said cells under skin growth-promoting conditions.

18. A method for increasing epidermal cell adhesion to a target surface, comprising coating said surface with at least one of the approximately 100 kD, 135 kD, 140 kD, 150 kD, or 400 kD proteins of the extracellular matrix deposited by the cell line 804G.

19. The method of Claim 18, wherein said cells are periodontal cells.

20. The method of Claim 18, wherein said cell adhesion occurs *ex vivo*.

21. The method of Claim 18, where said cell adhesion occurs *in vivo*.

22. A method for growing pancreatic islet cell precursors, comprising the step of culturing said cell precursors on matrix protein secreted by 804G cells.

23. The method of Claim 22, prior to the step of culturing, further comprising the steps of:

enzymatically digesting fetal pancreas; and

incubating the digested pancreas in cell culture medium until islet-like cell aggregates are formed.

24. The method of Claim 23, wherein said pancreas is from a mammal.

25. The method of Claim 24, wherein said pancreas is human.

26. The method of Claim 22, wherein said 804G matrix protein is attached to a substrate.

27. The method of Claim 22, wherein said matrix is produced by 804G rat bladder carcinoma cells.

28. A method for the generation of hormone-producing cells, comprising the steps of:

providing expanded endocrine precursor cells according to the method of Claim 1; and

transplanting said expanded endocrine precursor cells into a mammal.

29. The method of Claim 28, wherein said cells are pancreatic islet cells.

30. The method of Claim 28, wherein said hormone is insulin.

31. The method of Claim 29, wherein said cells are transplanted into or adjacent to the kidney, lung or liver.

32. Pancreatic islet cell precursors prepared in accordance with Claim 22.

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STATEMENT UNDER ARTICLE 19

Tsilibary et al. (US 5,007,925) disclose prosthetic devices coated with polypeptides having Type IV collagen activity useful in promoting cell attachment to substrates. Tsilibary et al. do not teach that these polypeptides induce hemidesmosome formation.

Riddelle et al. (*J. Cell Biol.*, 112:159-168, 1991) teach that 804G cells form hemidesmosomes when plated on a substrate. There is neither a teaching nor a suggestion that the matrix secreted by these cells induces hemidesmosome formation in other cells plated on the matrix. Riddelle et al. (*Mol. Biol. Cell*, 3:70a, 1992), Langhofer et al. (*Mol. Biol. Cell*, 70a, 1992) and Jones et al. (*J. Cell Biochem.*, 142:suppl. 16F, 1992) disclose description and localization of hemidesmosomal plaque components in 804G cells and

The instant invention relates to the formation of hemidesmosomes in cells plated on a matrix secreted by 804G rat bladder carcinoma cells. There is no teaching or suggestion to combine the cited references which would lead one of ordinary skill in the art to culture cells unable to form hemidesmosomes on the matrix secreted by 804G cells in order to stimulate hemidesmosome formation in the cultured cells.

Simpson et al. (*Diabetes*, 40:800-808, 1991) teach the growth of pancreatic ICCs from partially digested pancreas on bovine corneal matrix. The instant invention relates to culturing ICCs on 804G matrix. At page 26, lines 33-35, the specification indicates that superior results were obtained when the cells were cultured on 804G matrix as compared to bovine corneal matrix. Since there is no disclosure in the prior art that 804G matrix promotes better growth of endocrine cell precursors, the Simpson reference would not have provided motivation to the skilled artisan to culture the ICCs on 804G matrix since there would have been no advantage to doing so.

Thus, one of ordinary skill in the art would not have combined any of the cited references to arrive at the present invention. In view of the arguments presented above, Applicant submits that the claims patentably define over the prior art.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03733

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00; A61F 2/06, 2/10
US CL :435/240.2, 240.21, 240.23, 240.243; 623/1, 15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 240.23, 240.243; 623/1, 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A 5,007,925 (TSILIBARY ET AL) 16 April 1991, see entire document.	1-13, 17
Y,P	J. Cell Science, Vol. 105, issued July 1993, Langhofer et al, "The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro", page 753-764, see entire document.	1-13, 17, 22-28, 33-34
Y	J. Cell Biol., Vol. 112, No. 1, issued January 1991, Riddelle et al, "Formation od Hemidesmosomes In Vitro by a Transformed Rat Bladder Cell Line", pages 159-168, see entire document.	1-13, 17, 22-28, 33-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 JUNE 1994	JUL 05 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SUSAN M. DADIO Jill Warden for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Diabetes, Vol. 40, issued July 1991, Simpson et al, "Characterization of Endocrine-Rich Monolayers of Human Fetal Pancreas That Display Reduced Immunogenicity", pages 800-808, see entire document.	1-13, 17, 22-28, 33-34
Y	Mol. Biol. Cell., Vol. 3, issued 1992, Riddelle et al, "Substrate Attachment is Necessary for the Expression of Hemidesmosomal Proteins in Cultured Cells", page 70a, see abstract 404.	1-13, 17, 22-28, 33-34
Y	Mol. Biol. Cell., Vol. 3, issued 1992, Langhofer et al, "Matrix Signals Transduced by the alpha-686 Integrin Complex", page 95a, see abstract 550.	1-13, 17, 22-28, 33-34
Y	J. Cell Biochem., Vol 142, Suppl. 16F, issued 1992, "Alpha-6β4 Integrins: Their Role in the Assembly of the Hemidesmosome (HD) and in Signal Transduction", page 142, see abstract X 007.	1-13, 17, 22-28, 33-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03733

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-13, 17, 22-28 and 33-34

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/US94/03733

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-13, 17, drawn to an article of manufacture and method of generating skin by culturing epidermal cells onto the article, Class 435, Subclass 70.3 and 240.2, respectively.

II. Claims 14-15, drawn to a compositon and a protein, Class 530, Sublass 350.

III. Claim 16, drawn to an isolated polypeptide, Class 530, Subclass 350.

IV. Claims 18-21, drawn to a method for increasing cell adhesion to a target surface, Class 530, Subclass 350.

V. Claims 22-28 and 33-34, drawn to a method for growing endocrine precursor cells and the cells produced by the method, Class 435 240.2, Subclass 240.2.

VI. Claims 29-32, drawn to a method of generating hormone-producing cells, Class 424, Subclass 93U.

Group I, drawn to an article of manufacture is a materially distinct product from that of Group II drawn to a protein. Furthermore, both Groups I and II are materially distinct products from the polypeptide of Group III.

The method of increasing cell adhesion, Group IV, is a materially distinct process from that of Groups V and VI, drawn to growing endocrine cells and producing hormone-producing cells. Groups V and VI are materailly disctinct process in that they require completely different steps.

The Groups are not so linked by a special technical feature within the meaning of PCT Rule 13 so as to form a single general inventive concept and thus lack unity. See Pct Rules 13.1-13.4.